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TITLE:

The Role of ADAM9 in Tumor-Stromal Interactions in Breast Cancer

PRINCIPAL INVESTIGATOR:

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CONTRACTING ORGANIZATION:

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14. ABSTRACT **This final report covers the three years of work on the Statement of Work with particular focus on the fourth year no-cost extension, obtained for the purposes of finishing and publishing this work. This report covers the major accomplishments of this research, including the development of reagents to detect the secreted form of ADAM9, the development of a model system to investigate the isoforms of ADAM-9 in breast cancer cell migration, and the use of this model system to discover novel functions for both isoforms of ADAM9. We have confirmed the presence of the secreted form of ADAM9, ADAM9-S in breast cancer cell lines and tumor lysates,. We have also confirmed the role of ADAM9-S in promoting breast cancer cell migration and shown that this is through the proteolytic function of the protein. In addition, we have shown that the alternatively spliced, membrane-bound isoform of ADAM9, ADAM9-L, suppresses cell migration in a proteolysis-independent manner. These differences are significant as ADAM9 has been identified as a druggable target for breast cancer, and the potential side-effects of an ADAM9 inhibitor will depend on the functional domain targeted and isoform prevalence in disease.**			
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Introduction

The purpose of this research project is to evaluate the molecular mechanisms by which the isoforms of ADAM9 participate in breast cancer progression. Based on previous work [1], we hypothesized that ADAM9 participates in tumor-stromal interactions in breast cancer, acting to enhance tumor progression by mediating HB-EGF shedding and EGFR signaling, and participating in the epithelial-mesenchymal transition (EMT) through its integrin binding function. Our 2007-2009 annual summaries detailed the development of isoform specific ADAM9 antibodies and the specific detection of ADAM9 isoforms in breast cancer cell lines and tumor cell lysates. We also described the discovery of ADAM9-L suppression of breast cancer cell migration, and established that the protein cleavage function of the protein was not necessary to reconstitute suppression after protein silencing. This data led us to revise our statement of work to explore this mechanism and signaling, including the evaluation of functional domain mutants of ADAM9-L for their role in breast cancer cell migration. In addition, our studies have also shown that two isoforms of ADAM9 play opposing roles in breast cancer cell migration, and this report will summarize those results. The results of this research will provide us with a new mechanism by which the ADAM9 isoforms present in breast tumors and stroma influence cell migration, lending insight into tumor progression and metastasis. Increased understanding of these mechanisms will lead to new strategies for therapy targeted specifically to advanced stage tumors. Over the course of this grant this research has also prepared the Principal Investigator for her career in breast cancer research, including oral presentations at national meetings, poster presentations, and attendance at multiple symposia dedicated to breast cancer work. During the no-cost extension of this grant through 2010, the Principal Investigator obtained her Ph.D in Experimental Pathology from Harvard Medical School, and this work is currently in revisions as a publication in Cancer Research.

Body

The following tasks from the Statement of Work for this project were the focus for the research period from 31 March 2006 – 31 March 2010:

Task 1: To characterize the expression of ADAM9 in breast cancer tissues and cell lines representing different stages of the disease (months 1-13).

Task 2: Determine the role of ADAM9 in HB-EGF shedding and EGFR signaling in breast carcinoma cells (months 7-26).

Task 3: Evaluate the role of ADAM9 in the migration of breast carcinoma cells (months 24-36).

This final report will summarize the research conducted on each of these tasks as reported in previous annual reports, and describe in detail the results and subsequent experiments of the past 12 months. We have submitted this manuscript for publication in Cancer Research, and figures denoted Appendix reference the appended manuscript.

Task 1:

The goal of this Task is to develop immunohistochemical tests to determine the presence of the isoforms of ADAM9 in breast cancer cell lines and tissue arrays, and to ultimately evaluate the isoform distribution in the tumors and stroma of breast cancers with different clinical profiles. While Year one progress in this task included the development and testing of isoform-specific antibodies to both ADAM9-L and ADAM9-S (Appendix, Fig 1A,B), and the identification of breast cancer cell lines that endogenously express ADAM9-L and –S **(Appendix, Fig 1C)**. Year two research focused on using isoform specific antibodies for immunocytochemistry in preparation for staining tissue arrays. Unfortunately, we were not able to sufficiently lower the background to obtain signal for the ADAM9-S antibody for use in immunohistochemistry, rendering us unable to proceed to tissue arrays.

Current Data:

An ADAM9-S antibody suitable for immunohistochemistry is currently unavailable. To analyze the expression of ADAM9-S in patient samples, we obtained breast cancer tumor lysates from Dr. Gerburg Wulf [2] and ran them on an SDS-PAGE gel. Blotting with ADAM9-S specific antibody shows that ADAM9-S is expressed in tumor lysates as well as normal breast tissue **(Appendix, Fig1D)**.

Summary:

During the three years of research on this grant we have used our isoform-specific ADAM9 antibodies to show for the first time that ADAM9-S is expressed both in breast cancer cell lines and in patient samples. This proof of expression supports our work in Tasks 2 and 3, as determining the isoform specific migratory effects of ADAM9 is relevant to the study of breast cancer metastasis.

Task 2:

The overarching goal of Task 2 is to evaluate the role of ADAM9 in EGFR signaling in breast cancer cell lines, with the sub-goal of evaluating the role of the EGFR pathway in breast cancer cell migration/invasion. Due to our difficulty in originally evaluating breast cancer cell lines for ADAM9

isoform expression, and the promising preliminary migration data presented in our 2007 Annual Update and repeated here, we began this task with the evaluation of the role of ADAM9 in breast cancer cell migration. My original hypothesis, based on data from our lab in which both recombinant stimulation with ADAM9-S and transfected and expressed ADAM9-S enhances invasion of breast cancer cells through a matrigel-coated transwell[1], was that overexpression of ADAM9-S in breast cancer cells would enhance migration, and that overexpression of ADAM9-L would either replicate this phenotype or would have no migratory effect, indicating that the secreted form of ADAM9 was escaping regulation normally imparted through the cytoplasmic domain. Past annual summaries have described our data showing that ADAM9-L and –S have opposing migratory phenotypes and this story will be repeated here. This task further specifies that we will look at the EGFR pathway, as membrane bound EGF is the major known substrate of ADAM9 cleavage[3, 4], as the mechanism for action of ADAM9 mediated migration.

Current Data:

Overexpression of ADAM9 isoforms results in different migration phenotypes

Upon the discovery that both ADAM9 and ADAM9-S are expressed in migratory breast cancer cell lines (Task 1), PCDNA constructs of ADAM9-L, metalloprotease-deficient ADAM9L.EA, ADAM9-S, and ADAM9S.EA were transfected into BT549 breast cancer cells and their chemotactic migration evaluated using an uncoated transwell assay. Consistent with previous results [1], ADAM9-S overexpression causes a significant increase in migration, with no change seen in ADAM9-L overexpressing cells. Furthermore, overexpression of a metalloprotease-deficient ADAM9S.EA mutant has no migratory phenotype, indicating that the metalloprotease activity of ADAM9-S is responsible for increasing cell migration **(Appendix, Fig 2A)**.

ADAM9 Silencing Increases Breast Cancer Cell Migration

To evaluate the role of endogenous ADAM9 in cell migration, an siRNA oligonucleotide pool targeting various sequences in ADAM9 was purchased from Dharmicon Research Inc. Transient transfections of these oligonucleotides into BT549 cells, chosen for their high expression of ADAM9 and migratory capacity, resulted in a surprising increase in migration of the ADAM9-silenced cells through an uncoated transwell assay system **(Appendix, Fig 3A)**. To further confirm this phenotype, two lentiviral shRNA constructs were designed to target the 3' untranslated region of ADAM9 mRNA (ADAM9 shRNA.1) and the cysteine rich domain (ADAM9 shRNA.2). Silencing of ADAM9 by both sequences in BT549 cells resulted in an increase in migration in a transwell migration assay **(Appendix, Fig 3D)**. To exclude the possibility that our sequences were only silencing ADAM9-L we used lentiviral sequence #1 to knockdown ADAM9 in BT549 cells and blotted for ADAM9-S using

isoform specific antibody **(Appendix, Fig 3B)**. To confirm that ADAM9 silencing was responsible for the increase in migration phenotype, a reconstitution experiment was performed by introducing murine ADAM9-L via transient transfection for 24 hours after silencing of ADAM9 in BT549 cells using ADAM9 shRNA.1. A rescue of the phenotype is observed, implying a direct ADAM9-L phenotype correlation **(Appendix Fig 3C)**.

Silencing of both ADAM9 isoforms leads to an increase in cell migration, which is rescued by introduction of wild-type ADAM9-L. To determine the ability of ADAM9-S to contribute to the migration of cells in the presence or absence of endogenous ADAM9 isoforms, lentivirally-infected and selected BT549 cells were transfected with wild-type (hADAM9S) or metalloprotease-deficient (hADAM9S.EA) human ADAM9-S constructs. Consistent with previous literature[1], transfection of ADAM9-S into cells with endogenous ADAM9 results in an increase in cell migration, which is not seen upon overexpression of the metalloprotease-deficient mutant, implicating the metalloprotease domain in this mechanism **(Appendix, Figure 3C)**. Furthermore, the increase in migration is greatly enhanced upon the silencing of endogenous ADAM9, whereas the metalloprotease-deficient mutant behaves similarly to vector controls. These data indicates different and opposing roles for the two ADAM9 isoforms in breast cancer cell migration. At this point, we revised our statement of work to break this work on ADAM9 migration into two sections – task 2 encompasses work on ADAM9-S and the mechanism by which ADAM9-S is enhancing cell migration through its metalloprotease domain. Task 3 continues our work on the role of ADAM9-L in suppressing cell migration via functional domain studies.

ADAM9-S acts as a chemoattractant

To confirm that ADAM9-S is acting as an enhancer of cell migration through its metalloprotease activity and that overexpression is not causing an off-target phenotype we assayed the chemoattractant ability of ADAM9-S and metalloprotease-deficient ADAM9S.EA. First we overexpressed these constructs in HEK-293T cells, and collected serum-free supernatant containing ADAM9-S and ADAM9S.EA. This supernatant was then put in the bottom chamber of a transwell and BT549 breast cancer cells were placed in the top chamber of the transwell and allowed to migrate overnight. Due to the absence of serum in the chemoattractant medium, migration was not robust, but we can see a significant increase in the migration of BT549 cells migrating toward ADAM9-S containing medium in comparison to PCDNA and metalloprotease-deficient ADAM9S.EA medium **(Appendix, Fig 2B)**. This result, coupled with the earlier overexpression data, convincingly shows that ADAM9-S mediates breast cancer cell migration through its metalloprotease activity, and our

next step is to evaluate the known substrates of ADAM9, beginning with EGF for activity upon ADAM9-S stimulation.

ADAM9-S does not alter EGF signaling

To evaluate the activation of EGFR signaling by ADAM9-S, HEK293T cells were transfected with empty vector, ADAM9S.myc, and ADAM9S.EA.myc constructs. Media was changed post-transfection to serum-free media and this conditioned media was collected 48 hours post-transfection. Conditioned media was assayed for ADAM9-S expression using myc antibody. BT549 cells were serum-starved for 24 hours and then stimulated with ADAM9-S conditioned media or recombinant EGF **(Fig 1)**. Immunoblot with an EGFR antibody confirms equal loading of EGFR protein. Probing these blots with an anti-phospho-tyrosine antibody shows that ADAM9-S and ADAM9S.EA do not stimulate EGFR more than stimulation with recombinant EGF in serum-free medium or vector control, indicating that ADAM9-S does not enhance the phosphorylation of EGFR beyond the baseline stimulation by secreted factors from HEK293Ts.

Summary and Current Work

These studies support our initial hypothesis that ADAM9-S enhances the migration of breast cancer cell lines. By using both chemoattractant and overexpression studies we have shown this to be true despite evidence that suppression of migration by ADAM9-L is the predominant mechanism of ADAM9-mediated migration upon silencing in our system. This indicates that cells may switch the predominant isoform from ADAM9-L to ADAM9-S depending on mitigating signaling pathways in the cell. For example, ADAM9 transcription is responsive to oxidative stress [5], and ADAM9-L is a phosphorylation target of PKCδ [6]. Due to the small number of targets of ADAM9-S proteolytic cleavage, future work in this area will focus on an unbiased approach such as investigating the receptors activated in response to high levels of recombinant ADAM9-S using a phospho-receptor tyrosine kinase array (http://www.rndsystems.com/product_detail_objectname_rtkarray.aspx) or by isolating cleavage products from the conditioned media of ADAM9-S overexpressing or silenced cells and identifying them by mass-spectrometry.

Task 3 :

The goal of task 3 is to evaluate the role of ADAM9 in breast cancer cell migration. As outlined in the task 2 update, we have shown that the different isoforms of ADAM9 have opposing effects on breast cancer cell migration. ADAM9-S enhancing through its metalloprotease activity, and ADAM9-L suppressing via a mechanism we are exploring in this task. This task requires that we evaluate the

key functional domains of ADAM9-L, the metalloprotease, integrin-binding disintegrin, and cytoplasmic signaling domains for a role in breast cancer cell migration.

Current Data

Rescue of ADAM9 Silencing Using Functional Mutations

To determine the contribution of each functional domain of ADAM9 to the mechanism by which ADAM9 inhibits migration in a transwell assay, mutations in each functional domain were constructed in a murine ADAM9-L background to allow for rescue of lentiviral-induced silencing in BT549 cells **(Appendix, Fig 4A)**. BT549 cells infected with lentiviral vector alone experience no change in migration phenotype when overexpressing wild-type murine ADAM9-L (mA9L). However, when overexpressing the metalloprotease-deficient mA9L.EA, these cells experience an increase in migration. In the absence of endogenous ADAM-9, we see the increased migration phenotype expected in BT549 cells, which is rescued by wild-type mA9L. It is interesting that this phenotype is also rescued by mA9L.EA, indicating that the metalloprotease-deficient mutant may interfere with the mechanism of endogenous ADAM9-L, but in silenced conditions is able to act in lieu of the endogenous protein **(Appendix, Fig 4B)** This indicates that the metalloprotease activity is not critical to the function of ADAM9 in cell migration. Deletion of the cytoplasmic signaling domain (mA9L.delcyt) does not result in the rescue of the phenotype, indicating that cytoplasmic signaling is critical for the action of the protein **(Appendix, Fig 4C)** This points to the disintegrin domain as the major functional domain involved in migration, and current experiments are focused in this direction.

Assaying migration after gene reconstitution with the disintegrin domain mutant has proved to be impossible. Deletion of the entire domain results in the expression of a protein that cannot be processed in the golgi by furin **(Appendix, Fig 5A)**. In addition, we mutated the ECD amino acids which comprise the integrin-binding motif in ADAM disintegrin domains, which also results in failure of the protein to process appropriately **(Appendix, Fig 5A)**. This highlights a previously unknown critical role for the disintegrin-domain region of ADAM-9 in maintaining protein function.

To evaluate the role of integrin-binding in ADAM9-L mediated suppression of cancer cell migration, we used the α-6 integrin clustering antibody GOH3 to alter the adhesion properties of cells with and without ADAM9-L expression. Interestingly, the integrin-binding antibody enhanced the migration of cells with silenced ADAM9, and this phenotype was rescued by reconstitution of ADAM9-L expression **(Appendix, Fig 5B)**. This indicates that ADAM9-L is mediating integrin signaling, either at the integrin-level or by altering downstream signals.

Summary and Future Work

The progress on this task has led to the confirmation that ADAM9-L is suppressing breast cancer cell migration. Our original hypothesis that this would be via signaling initiated by the protein-cleavage function of the protein was disproven by the rescue of increased migration by addition of a metalloprotease-deficient mutant. Additional mutants of the functional regions of ADAM9-L have shown that the upstream or downstream signaling mediated by the cytoplasmic tail is critical for suppression of migration, and that the disintegrin domain is critical for proper protein processing. Evaluation of the disintegrin domain function using integrin-binding experiments revealed a connection between integrin interaction and the suppression of migration of ADAM9-L. Other possibilities are the cysteine-rich and EGF-like domains, however little is known about the function of these domains in ADAM9 or other ADAMs.

Key Research Accomplishments

- Our isoform specific antibody experiments show for the first time the expression of ADAM9-S in breast cancer cell lines and tumor lysates.
- Analysis of both isoforms of ADAM9 in cell migration shows that ADAM9-L and ADAM9-S have different and opposing roles in cell migration.
- Multiple breast cancer cell lines show enhanced migration upon the silencing of ADAM-9. This enhancement is eliminated when exogenous ADAM9-L is introduced into the silenced cell, confirming the specificity of this phenotype.
- Reconstitution of ADAM9-L signaling in BT549 cells by introduction of a metalloprotease-deficient ADAM9L.EA construct rescues the enhanced migration phenotype in silenced BT549s, indicating that cleavage of proteins are not the primary functions mediating cell migration.
- Altering integrin interactions in the context of ADAM9-deficient cells alters the migration phenotype seen in an ADAM9-dependent manner, indicating that the integrin-binding properties of ADAM9-L may play a role in suppression of cell migration.

Personnel Receiving Pay For This Research Effort:

Jessica L. Fry – Principal Investigator This research effort was the primary focus of the PI's graduate work and Curriculum Vitae, included in the appendix.

PI Training and Development (2009-2010)

In addition to the reportable outcomes of this project associated with the career development of the PI, the PI has also presented a poster at one departmental retreat and two national meeting as well as oral presentations of this work at departmental data clubs and 3 dissertation committee meetings.

This work also comprised the Ph.D dissertation of the PI, who successfully defended on November 19, 2009.

Reportable Outcomes (2006-2010) Meeting Abstracts/Publications

Comprehensive meetings and abstracts for the duration of the grant are listed on the PI's Curriculum Vitae, available in the appendix. The PI also obtained her Ph.D in Experimental Pathology from Harvard Medical School based on the work done for this award. The PI is also currently exploring job opportunities in academic science.

Conclusion

This final summary encompasses the entirety of the Statement of Work for this project. This project marks the first time the secreted isoform of ADAM9, ADAM9-S, has been detected endogenously, specifically in breast cancer tumors and cell lines. The development of a lentiviral shRNA system for silencing ADAM9 allows us to silence endogenous ADAM-9 in human breast cancer cell lines and to identify the migration phenotype. Our results indicate that ADAM9-L suppresses migration of breast cancer cells, while ADAM9-S increases cell migration. Reconstitution experiments using wild-type ADAM9-L eliminate this phenotype, while ADAM9-S enhances migration in both the presence and absence of both ADAM9 isoforms, which is analogous to what was observed in overexpression experiments. We are also the first to propose a mechanism for ADAM9-S function in cell migration, via the metalloprotease activity, and we build upon work in the field linking the disintegrin domain of ADAM9-L to migration in other cell types [7]. This detailed work is especially important given the identification of ADAM9 as a potential target of breast cancer therapy [8]. Studies that consider the functional role of each isoform in different genotypic contexts, *in vitro* as well as *in vivo*, will be important for the development of safe and effective treatments that target both tumorigenesis and metastasis. Our work has lent insight into the functions and putative mechanisms of both isoforms of ADAM-9, and our future work in this area will further define the relevant signaling pathways which participate in ADAM9 mediated cell migration, paving the way for future studies and possible interventions in breast cancer invasion and metastasis.

References:

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3. Blobel, C.P., *ADAMs: key components in EGFR signalling and development.* Nat Rev Mol Cell Biol, 2005. **6**(1): p. 32-43.
4. Peduto, L., et al., *Critical function for ADAM9 in mouse prostate cancer.* Cancer Res, 2005. **65**(20): p. 9312-9.
5. Sung, S.Y., et al., *Oxidative Stress Induces ADAM9 Protein Expression in Human Prostate Cancer Cells.* Cancer Res, 2006. **66**(19): p. 9519-26.

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7. Zigrino, P., et al., *Role of ADAM-9 disintegrin-cysteine-rich domains in human keratinocyte migration.* J Biol Chem, 2007. **282**(42): p. 30785-93.
8. Neve, R.M., et al., *A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes.* Cancer Cell, 2006. **10**(6): p. 515-27.

Figure 1.

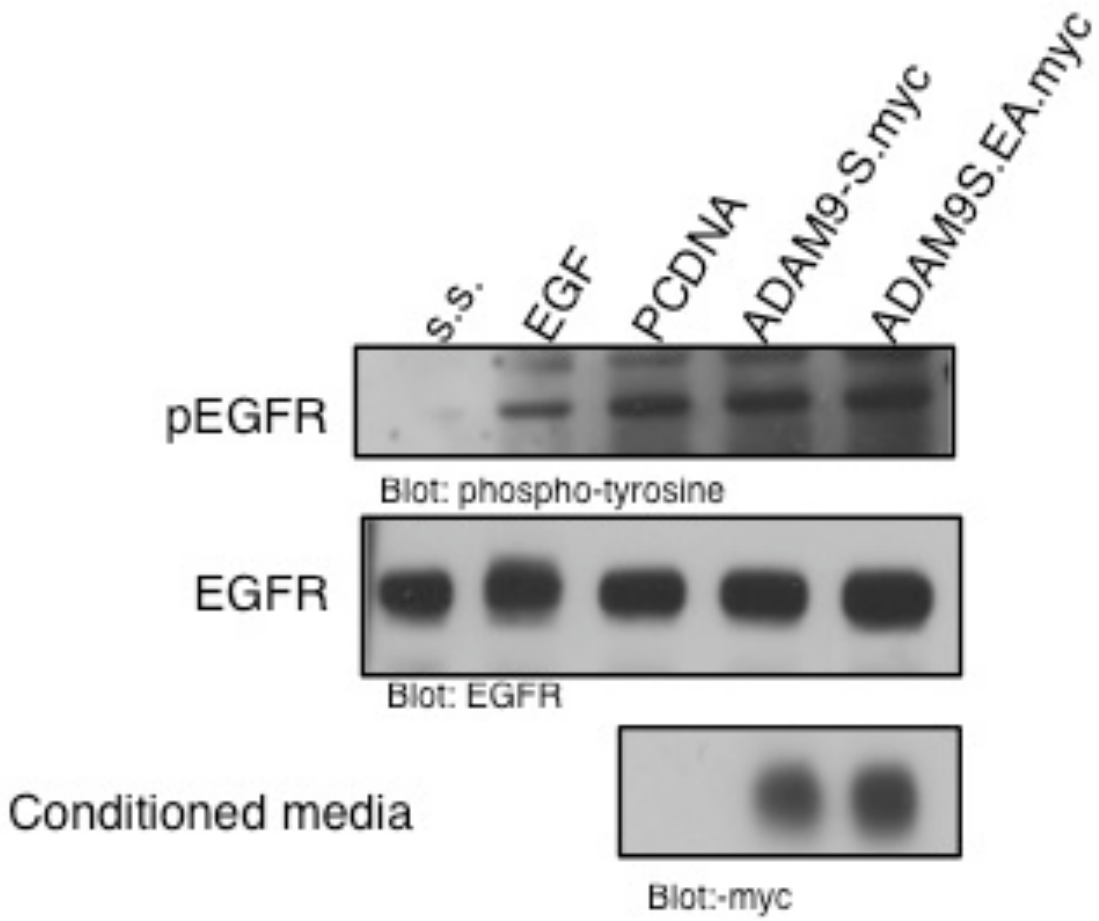

Figure 1 ADAM9-S stimulation of EGFR Serum-free supernatant from HEK293T cells overexpressing PCDNA control, ADAM9S.myc, or ADAM9S.EA.myc was used to stimulate BT549 cells for 15 minutes prior to lysis in NP40 lysis buffer. Serum starved (s.s.) and cells treated with 100uM EGF were used to confirm phosphorylation. Lysates were run on an 8% SDS-PAGE gel, and blotted for EGFR and phospho-tyrosine. HEK293T supernatant was collected and blotted with α-myc to confirm ADAM9-S construct expression.

Appendix I Curriculum Vitae, Jessica L. Fry Ph.D

Jessica L. Fry

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EDUCATION

Harvard University, Division of Medical Sciences — Boston, MA
Ph.D. Experimental Pathology, Program in Biological and Biomedical Sciences — November 2009
Dissertation: ADAM9 Metalloproteases and Breast Cancer Cell Migration

Massachusetts Institute of Technology — Cambridge, MA
S.B. Biology — 2003

RESEARCH EXPERIENCE

Harvard University, Beth Israel Deaconess Medical Center — Boston, MA
Graduate Researcher; Advisor: Alex Toker, Ph.D. — 2005-2010

The differential roles of A Disintegrin and Metalloprotease (ADAM)-9 isoforms in breast cancer cell migration and invasion.

- Confirmed the expression of the secreted isoform of ADAM9 (ADAM9-S) in breast cancer cell lines and tumors and the pro-migratory function of this protein.
- Identified opposing roles for membrane-bound ADAM9-L and ADAM9-S in cancer cell migration
- Tested the role of the metalloprotease function of ADAM9-S in cell migration.
- Explored the mechanism by which ADAM9-L suppresses cell migration using gene reconstitution experiments and mutants in key functional domains, peptide blocking of the integrin-binding domain, and basic biochemistry.

Massachusetts Institute of Technology — Cambridge, MA
Undergraduate Researcher; Advisor: Tyler Jacks, Ph.D. — 2001-2003

Characterization of a GFP fusion to the N-terminus of the Neurofibromatosis Type 2 tumor suppressor protein, Merlin.

Techniques/Skills*:*
Retroviral vector construction and infection, flow cytometry, tissue culture and transfection, murine colony organization and husbandry, murine embryonic fibroblast collection, tumorigenicity assays – proliferation, focus formation, soft agar colony formation, and tumor formation in nude mice, co-immunoprecipitation, Western blotting, molecular biology techniques such as site-directed mutagenesis and cloning.

GRANTS AND AWARDS

Pre-Doctoral Traineeship Award — 2006-2009
Department of Defense Breast Cancer Research Program

ASBMB Poster Award – FASEB Experimental Biology 2009

PUBLICATIONS

Research articles

Fry, J.L., Toker, A. (2009). The two isoforms of ADAM9 have opposing functions in breast cancer cell migration. (In Review, Cancer Research)

Johnson, K.C., Kissil, J.L., **Fry, J.L.**, and Jacks, T. (2002). Cellular transformation by a FERM domain mutant of the Nf2 tumor suppressor gene. Oncogene 21, 5990-7

CONFERENCE PRESENTATIONS

Oral Presentations

- **Department of Defense Breast Cancer Research Program Meeting**
  Era of Hope, Baltimore, MD, June 2008
  Fry, JL, Toker A. The role of ADAM-9 in breast cancer cell migration.

Poster Presentations

- **American Association of Cancer Research - Frontiers in Basic Cancer Research**
  Boston, MA October 2009
  Jessica L. Fry, Alex Toker The two isoforms of ADAM9 have opposing functions in breast cancer cell migration.

- **FASEB – Experimental Biology 2009**
  ASBMB Principles of Receptor Signaling, New Orleans, LA, April 2009
  J.L Fry, A.Toker ADAM9 isoforms in breast cancer cell migration.

- **Dana-Farber/Harvard Cancer Center - Program in Breast Cancer**
  Sixth Annual Symposium, Boston, MA, March 2009
  Jessica L. Fry and Alex Toker The two isoforms of ADAM9 differentially modulate breast cancer cell migration.

- **Beth Israel Deaconess Cancer Center Symposium**
  Defining New Frontiers to Eradicate Cancer, Boston, MA, October 2008
  Fry, JL, Toker A. The role of ADAM-9 in breast cancer cell migration.

- **Department of Defense Breast Cancer Research Program Meeting**
  Era of Hope, Baltimore, MD, June 2008
  Fry, JL, Toker A. The role of ADAM-9 in breast cancer cell migration.

TEACHING EXPERIENCE

Harvard University	Boston, MA
Teaching Assistant, Pathology 211	2005
Mentor, Mentoring for Science program	2005
Tutor, Cell Biology 201	2005-present

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APPENDIX II Submitted Manuscript

Secreted and Membrane-bound Isoforms of ADAM9 Have Opposing Effects on Breast Cancer Cell Migration.

Jessica L. Fry and Alex Toker

Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School Boston, Massachusetts, 02215

Running Title: ADAM9 isoforms in breast cancer cell migration

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Key Words: ADAM9, cell migration, breast cancer, metalloprotease, disintegrin

Abstract

Tumor cell migration is mediated by cell autonomous signaling mechanisms as well as paracrine and autocrine factors secreted by activated stromal cells in the tumor microenvironment. As a member of the ADAM family, the integrin-binding zinc metalloprotease ADAM9 modulates cell-cell and cell-matrix interactions as well as ectodomain shedding of cell surface receptors and ligands, thereby modifying intracellular and extracellular signaling. ADAM9 transcripts are alternatively spliced to express a transmembrane protein (ADAM9-L) and a secreted variant (ADAM9-S). In this study, we show that ADAM9-S promotes breast cancer cell migration in a manner requiring its metalloprotease activity, whereas ADAM9-L suppresses cell migration independent of its metalloprotease activity. Suppression of migration by ADAM9-L requires a functional cytoplasmic domain and integrin binding. We also reveal expression of both isoforms in breast cancer cell lines and tissues. Therefore, the expression of membrane-tethered and secreted variants of ADAM9 may be a key determinant in the aggressive migratory phenotypes associated with breast cancer progression.

Introduction

One of the hallmarks of advanced breast cancer is the ability of the tumor cells to lose their epithelial phenotype, disengage from neighboring cells, degrade the basement membrane and invade surrounding tissues, ultimately metastasizing to distant organs. The interactions between the tumor and its stromal microenvironment is a key determinant in breast cancer progression from carcinoma in situ to advanced invasive and metastatic carcinoma. Tumor cells proteolyze the basement membrane and stroma to invade the vasculature, and also alter stromal signals to stimulate angiogenesis and alternately release and tether to the extracellular matrix (ECM) to allow for efficient migration. Tumor cells also release growth factors and chemokines that alter the stroma, inducing inflammation, angiogenesis and mechanisms of tissue repair (1, 2).

Proteases play a major signaling role at the tumor-stromal boundary. Enzymes comprising the matrix metalloprotease (MMP) family proteolyze basement membrane and function in pro-migratory signaling mechanisms. Proteolysis by MMPs exposes cryptic sites in ECM constituents such as laminin-5 and collagen IV that in turn promote tumor cell migration (3, 4). Proteolysis of the basement membrane also releases ECM-bound growth factors such as insulin and fibroblast growth factor (FGF) (5, 6). MMPs proteolytically cleave and release the ectodomains of multiple signaling molecules from the cell membrane in a process known as membrane shedding (7). Shedding of substrates such as transforming growth factor-β (TGFβ) (8) and tumor necrosis factor-α (TNFα) (9), activates signaling pathways that promote cell migration and survival. The ADAM (A Disintegrin and Metalloprotease) family of proteases has functional similarity to the MMPs in their zinc-binding metalloprotease domains, and are also referred to as the metalloprotease, disintegrin, cysteine-rich (MDC) family. ADAMs control basement membrane proteolysis and shedding of proteins from the cell membrane. Comprising 40 isoforms, the ADAM family of proteases also contain an integrin-binding disintegrin domain. ADAMs function in cell adhesion through their cysteine-rich domains that bind to Syndecans (10) and fibronectin (11). The Src homology-3 (SH3) binding domain in the cytoplasmic regions of some ADAMs mediate signaling by activation of Src and Grb proteins (12). Recent studies point to a functional importance for some ADAM family members in cancer. ADAM12 is expressed in carcinoma and promotes breast cancer progression by inducing the apoptosis of surrounding stromal cells (13). Consequently, ADAM12 protein levels correlate with advanced breast cancer (14, 15). In contrast, the disintegrin domain of ADAM15 inhibits angiogenesis and tumor growth (16). Moreover, ADAM15 expression decreases integrin-mediated ovarian cancer cell adhesion and motility (17).

ADAM9, also known as MDC9 (metalloprotease/disintegrin/cysteine-rich protein 9), MCMP (Myeloma cell metalloprotease) and meltrin-gamma, comprises a subgroup of ADAMs that also contains ADAM12 and ADAM15 (18). The ADAM9 metalloprotease activity cleaves heparin-binding epidermal growth factor (HB-EGF) (19), which binds activates EGF-receptor to promote tumor growth and angiogenesis (20). ADAM9 also cleaves Delta-like 1, a Notch ligand

(21), as well as ADAM10 (22, 23). The ADAM9 disintegrin domain is a ligand for specific integrin heterodimers including multiple β1 integrins (24, 25), α6β4 (26) and $\alpha_v\beta_5$ integrins (27, 28).

Recent studies have revealed that ADAM9 message is upregulated in breast tumors as well as breast cancer cell lines (29, 30). The chromosomal region of the *ADAM9* gene (8p11-12) is amplified in a number of breast cancers and cell lines, and ADAM9 is overexpressed in cell lines encompassing the luminal, basal A, and basal B gene expression clusters (31).

The *ADAM9* transcript is alternatively spliced into membrane-bound and secreted isoforms. Alternative splicing of exon 12 in the ADAM9 message leads to a shorter ADAM9-S (ADAM9-secreted) transcript that contains eight unique amino acids not present in ADAM9-L (ADAM9-long). ADAM9-S also lacks the transmembrane and cytoplasmic domains and is a secreted protein (26). ADAM9 is implicated in cancer cell phenotypes as secretion of ADAM9-S promotes invasion of breast cancer cells in Matrigel assays (26). Furthermore, adhesion of keratinocyte cells to recombinant ADAM9 disintegrin-cysteine rich domain increases cell migration, and overexpression of ADAM9 also increases pro-MMP9 expression (25). However, the functional role of ADAM9-L and ADAM9-S in modulating cell migration and invasion in breast cancer cells has not been evaluated.

Here, we use specific ADAM9-L and ADAM9-S antibodies to detect expression of both variants in breast cancer cell lines and breast cancer tissues. Using RNA interference and gene replacement, we show that ADAM9-S and ADAM9-L do not function in a redundant manner in the regulation of cell migration, whereby ADAM9-S promotes and ADAM9-L attenuates migration. These studies identify for the first time an ADAM as a migration suppressor, and have implications for the functional roles of ADAM9 proteins in breast cancer progression.

Materials and Methods

Antibodies and Reagents

Anti-myc antibody purified from the 9B11 hybridoma was purchased from Cell Signaling Technology (Danver, MA). Anti-β-actin was from Sigma-Aldrich (St Louis, Missouri). Anti-ADAM9-L was from BioMol Research Laboratories Inc (SA-376). Anti-ADAM9-S was designed and produced in collaboration with Pro-Sci Inc. (Poway, CA) as a rabbit-polyclonal antibody based on the immunization peptide sequence CATGLSLKFHAPF. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) or polyethyleimine (Polysciences Inc). pcDNA3-mA9L, pcDNA3-mA9L.EA, pcDNA3-mADAM9L.myc and pcDNA4-hADAM9S.myc have been described previously (12, 26). Point mutants mADAM9L.EA.myc, ADAM9S.EA.myc and ADAM9L.TCE.myc were derived from these vectors via Stratagene site-directed mutagenesis according to the manufacturers instructions and the following primers: ADAM9S.EA 5' CCAAGATTATGACCCAATGCATGAGCAACAATGG 3' and 5' CCATTGTTGCTCATGCATTGGGTCATAATCTTGG 3'; mADAM9L.EA 5' CCATTGTTGCTCATGCATTGGGGCATAACCTTGG 3' and 5' CCAAGGTTATGCCCCAATGCATGAGCAACAATGG 3'; mADAM9L.TCE.myc 5' GGACCCTGGAGAGCGTGTGAATGCGGC 3' and 5' GCCGCATTCACACGTCTCTCCAGGGTCC 3'. Deletion mutations in mADAM9L.Δcyt.myc and mADAM9L.Δdis.myc were derived as follows: To delete the cytoplasmic domain, a KPN1 restriction site was inserted into pcDNA3-mADAM9-L 5' of the cytoplasmic region with primers 5' GGCTAACTAGAGAACCCACTGGTACCTGGCTTATCG 3' and 5' CGATAAGCCAGGTACCAGTGGGTTCTCTAGTTAGCC 3'. BAMHI from the vector backbone and the inserted KPN1 restriction sites were used to isolate the mADAM9L.Δcyt sequence, which was cloned into digested pCDNA4A/TO/myc/his vector in frame with the Myc tag. To delete the disintegrin domain, KPN1 sites were inserted 5' and 3' of the disintegrin domain in pCDNA3-mADAM9L.myc using site-directed mutagenesis using the following sequences : 5' KPN1 5' GTGGAGCAAAGAGCGGTACCATGAATTCAGGAGC 3' and 5' GCTCCTGAATTCATGGTACCGCTCTTTGCTCCAC 3'; 3' KPN1 5' GAAGGAGTGTGAGGGTACCCCATGCTGTGAAGGAAG 3' and 5' CTTCCTTCACAGCATGGGGTACCCTCACACTCCTTC 3'. KPN1 digestion was used to isolate and discard the disintegrin sequence, and the vector backbone KPN1 sites were ligated. For RNAi (RNA interference), ADAM9 sequences from the Mission siRNA project (Sigma Aldrich) were cloned into the pLKO.1 vector directing expression of short hairpin RNA (shRNA) (32). The following sequences were used: ADAM9shRNA.1 Sense 5' CCGGCCCAGAGAAGTTCCTATATATCTCGAGATATATAGGAACTTCTCTGGGTTTTTG 3' antisense: 5' AATTCAAAAACCCAGAGAAGTTCCTATATATCTCGAGATATATAGGAACTTCTCTGG 3'; ADAM9shRNA.2 Sense 5'

CCGGGCCAGAATAACAAAGCCTATTCTCGAGAATAGGCTTTGTTATTCTGGCTTT 3' antisense 5' AATTCAAAAAGCCAGAATAACAAAGCCTATTCTCGAGAATAGGCTTTGTTATTCTGGC 3'. Second-generation lentiviral packaging plasmids pCMV-dR8.2 dvpr and pCMV-VSVG were obtained from ADDGENE (Cambridge, MA) for lentiviral packaging.

Cell Lines

All cell lines were obtained from ATCC, with the exception of the estrogen-independent human breast cancer SUM-159-PT which has been described (33). BT549, HCC38, and ZR75-1 cells were maintained in RPMI supplemented with 10% fetal bovine serum. MDA-MB-231, HEK293T, MDA-MB-468 and NIH3T3 cells were maintained in DMEM supplemented with 10% fetal bovine serum. CAMA-1 cells were maintained in MEM supplemented with 10% fetal bovine serum. SKBR3 cells were maintained in McCoy's Modified Medium 5a supplemented with 10% fetal bovine serum. MCF10A cells were maintained in DMEM modified with 5% equine serum, 20ng/ml EGF, 10ug/ml insulin, 100ng/ml cholera toxin, 500ng/ml hydrocortisone, and 1% penicillin and streptomycin.

Cell Lysis

Cells were lysed in NP40 lysis buffer (20mM Tris HCl [pH 7.0], 10% glycerol, 1% NP40, 10mM EDTA, 150mM NaCl, 20mM NaF, 5mM sodium pyrophosphate) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was calculated using Bio-Rad Protein Assay solution

Immunoprecipitation

Equal amounts of protein were incubated at 4°C in a 1mL volume with 2µg antibody for 4 h prior to addition of Protein A Sepharose beads. Beads were incubated for 2 h, and then washed with 1% NP40 in PBS three times before elution with 2x Laemmli lysis buffer (125mM Tris [pH6.8] , 23% Glycerol, 4% SDS, 10% β-mercaptoethanol, bromphenol blue) and immunoblotting.

Immunoblotting

Cell lysates were equilibrated using the BioRad Protein Assay solution, and 50-100 µg protein was resolved by SDS PAGE, transferred to nitrocellulose (Hoefer semi-dry transfer system, 160mA, 80-120 min), and blocked in 5% non-fat milk in 1%TBST (1% Tween in TBS). Membranes were incubated with the appropriate antibodies (primary antibodies used at the concentration recommended by manufacturer, ADAM9-S used at 2µg/mL) for 14 h. Cell membranes were

washed in 1%TBST and incubated with peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Jackson Immunoresearch) and exposed using high-sensitivity ECL (Millipore).

Lentiviral Infection

pLKO.1 plasmid constructs were co-transfected into HEK293T cells with packaging vectors pCMV-dR8.2 dvpr and pCMV-VSVG using polyethyleimine. 48 h post-transfection, lentiviral particles in supernatant were harvested, passed through a 0.45μM filter and either stored at -80°C or used to infect target cells. Target cells at approximately 80% confluency were infected with virus alone for a period of 4 h at which point they were supplemented with full serum and allowed to recover for 24 h before selection in puromycin in full culture medium for 18 h (2-5 μg/mL).

Transfection of siRNA into BT549 cells.

pCS2-(n)-β-galactosidase reporter plasmid (1μg) was transfected alone, or with 200pM of non-targeting or ADAM9 siRNA pools according to the Lipofectamine 2000 protocol (5μl Lipofectamine 2000, 500μl Optimem (Gibco)) into $5x10^5$ BT549 cells per 6cm dish (Corning). Media was replaced with full-serum medium 4 h post-transfection.

Transfection of plasmid DNA.

Plasmids of interest and the pCS2-(n)-β-galactosidase reporter plasmid were co-transfected at a ratio of 6.5:1 into non-infected or lentivirally-infected cells 24 h post-infection with Lipofectamine 2000 according to manufacturers' instructions. Media was replaced with full culture medium for non-infected cells or puromycin selection media 4 h post-transfection. Cells were assayed for migration and protein expression 24 h post-transfection.

Migration Assays

Migration assays were carried out using Transwell chambers (Corning, Acton, Massachusetts) with 8 μM pore membranes. Cells were trypsinized and resuspended in serum-free media containing 0.1% BSA. $1x10^5$ cells were added to the top of each Transwell chamber, and allowed to migrate toward cell-conditioned media for 4-16 h at 37°C depending on the cell type. Cells that had migrated to the lower surface of the membrane were fixed and stained. Lentivirally-infected cells were stained with Hema-3 Protocol Stain (Fisher Scientific). Cells co-transfected with expression plasmids and β-galactosidase were stained with X-galactosidase. Experiments were conducted in triplicate, and equal fields of stained cells were counted in each well and normalized to the plating efficiency or the transfection efficiency. Transfection efficiency was calculated by counting the number of transfected cells per 100,000. Plating efficiency was calculated by counting two wells of 100,000 cells plated in parallel to the migration assays to control for minor discrepancies in plating.

Statistical analysis was performed using ANOVA and the InStat statistical analysis software. Cell-conditioned media was harvested from NIH3T3 fibroblast cells grown in full-serum for 48 h to confluency, or HEK293T cells transfected with ADAM9-S expression plasmids and incubated in serum-free media for 48 h.

Integrin-blocking assays

Cells were infected and transfected with plasmid DNA as described above. Prior to migration assay cells were trypsinized and incubated with 5μg/ml of GOH3 antibody for 10 min at room temperature.

Results

ADAM9-S is expressed in breast cancer cell lines and breast tumors.

We first determined the expression pattern of ADAM9-S and ADAM9-L in breast cancer cells and breast cancer tissues. To detect specific endogenous isoforms of ADAM-9, a polyclonal antibody directed toward the carboxyl-terminus of ADAM9-L and an ADAM9-S specific polyclonal antibody designed against the unique carboxyl-terminus of ADAM9-S were used (Fig. 1*A*). To confirm isoform specificity, Myc-tagged murine ADAM9-L and human ADAM9-S were transiently expressed in HEK-293T cells. Equal amounts of protein were immunoblotted with Myc antibody to validate equivalent expression and ADAM9 antibody specificity. The results show that as expected, ADAM9-L is detected by Myc and ADAM9-L antibodies as a pro-ADAM9-L variant that comprises the unprocessed form migrating at 114kDa, and the fully processed mature membrane bound form at 84kDa (Fig. 1*B*). Expression of ADAM9-S is detected by Myc and ADAM9-S antibodies at 67kDa, as previously shown (26). Importantly, the ADAM9-L antibody does not cross-react with ADAM9-S, and the ADAM9-S antibody does not detect ADAM9-L (Fig. 1*B*). Therefore, specific antibodies against ADAM9-L and ADAM9-S are specific to the secreted and membrane bound variants of ADAM9. It is important to note that ADAM9-L and ADAM9-S migrate slower on SDS PAGE than their predicted molecular mass due to the presence of cysteine-rich regions as well as glycosylation.

To evaluate the expression of ADAM9-S and ADAM9-L in breast cancer cell lines, lysates were collected from BT549, HCC38, MDA-MB-231, SUM159PT, MCF10A, MDA-MB-468, CAMA-1, ZR75-1, MCF-7, and SKBR-3 cells at approximately 80-90% confluence, and ADAM9 expression was determined by immunoblotting. ADAM9-L expression is detected in all cell lines tested (Fig. 1*C dark exposure*). Importantly, cells previously characterized to overexpress ADAM9 (BT549, HCC38, CAMA-1) (31) reveal a substantially higher level of expression of ADAM9-L compared to other lines (Fig 1*C light exposure*). In addition, the variance in ADAM9-L bands detected by immunoblotting reflects differences in processing as well as post-translational modifications that include proteolytic cleavage and glycosylation. ADAM9-S endogenous expression is detected in BT549, HCC38, SUM159PT, MCF10A, and MDA-MB-468 cells (Fig 1*C*). Thus, ADAM9-L endogenous expression is detected in cells of both the luminal and basal breast cancer subtypes, while ADAM9S is predominantly observed in basal-type cells. Notably, CAMA-1, a non-invasive cell type, expresses ADAM9-L, but not ADAM9-S.

To evaluate expression of ADAM9 isoforms in breast cancer tissues, ADAM9-S levels were evaluated in Stage II and III invasive ductal carcinoma tumor and normal breast lysate samples kindly provided by Dr. Gerburg Wulf (Beth Israel Deaconess Medical Center) (34). The results show that ADAM9-S is expressed in breast cancers of both grades as well as normal tissue (Fig 1*D*).

Overexpression of ADAM9-S promotes breast cancer cell migration. Previous studies have shown that cancer cells exhibit increased translocation through Matrigel in the presence of exogenous recombinant ADAM9-S (26). To evaluate the role of ADAM9 isoforms in cell migration independent of matrix proteolysis, motility was assayed using uncoated Transwells. Plasmid constructs directing the expression of wild type and mutant murine ADAM9-L and human ADAM9-S were transfected into BT549 breast cancer cells. Overexpression of wild-type ADAM9-S significantly increases BT549 migration. In contrast, overexpression of a metalloprotease-deficient ADAM9-S.EA mutant with a Glu to Ala substitution in the metalloprotease active site (35) does not increase migration and is comparable to control transfected cells (Fig. 2*A*). Similarly, overexpression of the ADAM9-L variant did not promote BT549 cell migration, nor did overexpression of the equivalent metalloprotease-deficient ADAM9L.EA mutant. Therefore, as previously reported, ADAM9-S promotes cell migration in a protease activity-dependent manner. However, this phenotype is not recapitulated by ADAM9-L.

To confirm that extracellular ADAM9-S stimulates cell migration, ADAM9-S, ADAM9S.EA or vector control was expressed in HEK293T cells and serum-free conditioned media was collected post-transfection. These supernatants were added to the bottom chamber of uncoated transwells, and BT549 breast cancer cells were added to the top chamber. Under these conditions, BT549 cells require an extended 16 hour incubation period to migrate. However, BT549 cells exhibit increased migration when exposed to ADAM9-S-containing conditioned media in comparison to control-transfected or ADAM9S.EA-transfected media, indicating that exogenous ADAM9-S stimulates haptotactic migration through its metalloprotease activity (Fig 2*B*).

ADAM9 silencing increases breast cancer cell migration. To determine the functional role of endogenous ADAM9 in cell migration, we preformed loss of function experiments using an siRNA oligonucleotide pool targeting five distinct sequences spanning both ADAM9 isoforms. We chose to transiently transfect these siRNA's into BT549 cells, which express high levels of ADAM9 and are also highly migratory. Surprisingly, ADAM9 siRNA increases migration in BT549 cells as measured by Transwell migration assays, compared to control or non-target siRNA (Fig. 3*A*). This is concomitant with quantitative silencing of ADAM9-L protein expression. The phenotype of increased migration by ADAM9 siRNA recapitulates that observed upon expression of ADAM9-S (Fig. 2*A*). To evaluate that this is not due to an off-target effect of the siRNA pool, two distinct lentiviral shRNA constructs were generated to target both isoforms in the 3' untranslated region of ADAM9 mRNA (ADAM9 shRNA.1) and the cysteine-rich domain (ADAM9 shRNA.2). After lentiviral infection and selection, robust silencing of ADAM9-L and ADAM9-S protein is observed in cells transduced with both sequences as revealed by immunoblotting (Fig. 3*B*). Importantly, transduction of both shRNA sequences also results in an increase in

BT549 cell migration (Fig. 3*D*). Moreover, increased cell migration resulting from transduction of ADAM9 shRNA is also reproduced in distinct breast cancer cell lines, including CAMA-1 and ZR75-1 (Fig. 3*D*). Therefore, in different luminal and basal breast cancer type cells silencing ADAM9 expression results in enhanced cell migration observed with ADAM9 shRNA.1 and .2 sequences.

To further confirm that ADAM9 silencing is responsible for the increase in migration observed with shRNA, a reconstitution experiment was performed by introducing murine ADAM9-L and human ADAM9-S in cells harboring ADAM9 shRNA. The murine ADAM9-L (mADAM9-L) would be predicted to be refractory to silencing using human ADAM9. Consistent with this, expression of mADAM9-L protein was resistant to silencing in cells expressing ADAM9 shRNA, compared to cells with ADAM9 shRNA alone that show a robust reduction of ADAM9-L protein (Fig. 3*C*). In Transwell assays, expression of mADAM9-L rescues the increase in migration observed with shRNA alone. Therefore, the increase in migration observed upon silencing of ADAM9 is specific to ADAM9-L expression and is not due to off-target effects. The implication is that endogenous ADAM9-L is a suppressor of migration of breast cancer cells.

We also evaluated the contribution of ADAM9-S in the enhanced migration phenotype observed with ADAM9 shRNA. As observed in Fig. 2A, expression of wild type, but not protease-deficient ADAM9-S increases cell migration in control BT549 cells (Fig. 3*C*). In contrast to the effect of expression of mADAM9-L, the increase in migration induced by ADAM9 silencing is not rescued by expression of ADAM9-S that is refractory to silencing as the shRNA used (ADAM9 shRNA.1) targets the 3' untranslated region. Instead, expression of ADAM9-S leads to a further increase in the migratory phenotype that is additive to ADAM9 shRNA and ADAM9-S. As expected, this additive effect is not recapitulated by expression of the ADAM9-S protease inactive EA mutant (Fig. 3*C*). Therefore, ADAM9-S does not phenocopy the migration suppression function of ADAM9-L, and moreover the implication from these results is that suppression of migration is the dominant role of ADAM9 in breast cancer cells.

Functional role of ADAM9-L domains in suppression of cell migration. The data thus far demonstrate that ADAM9-S is an enhancer of cell migration, whereas ADAM9-L is a suppressor of migration. We next determined the contribution of the ADAM9-L functional domains in the suppression of migration. We generated mutations or deletions in the metalloprotease (ADAM9-L.EA), disintegrin (ADAM9-L.ΔDis) and cytoplasmic (ADAM9-L.ΔCyt) domains (Fig. 4*A*). Again the murine ADAM9-L allele was used to generate these mutations as it is refractory to silencing in human breast cancer cells. Wild type and mutant mADAM9-L alleles were introduced into BT549 cells subsequent to silencing with ADAM9 shRNA. As already observed, expression of the wild type ADAM9-L allele rescued the phenotype of increased migration upon ADAM9 silencing (Fig. 4*B*). This was also observed in cells expressing the metalloprotease-deficient ADAM9-L.EA mutant, indicating that the proteolytic activity of ADAM9-L is dispensable for the suppression of migration. In

contrast, there was no rescue of increased migration with the cytoplasmic domain deletion mutant (ADAM9-L.ΔCyt), demonstrating that localization and downstream signaling mediated by the ADAM9-L cytoplasmic domain is required for suppression of cell migration (Fig. 4*C*).

To address the role of the ADAM9-L disintegrin domain and integrin-binding activity, point mutations in the ECD motif as well as whole deletions in the integrin-binding domain were generated. However, expression of these mutant proteins followed by immunoblotting revealed that both mutants are not properly processed from the pro-domain containing form (114 kDa) to the active and mature 84kDa form, compared to wild type ADAM9-L (Fig. 5*A*). Therefore, we assessed the contribution of the disintegrin domain in the regulation of cell migration using integrin function blocking antibodies. BT549 cells were transduced with ADAM9 shRNA and reconstituted with wild type murine ADAM9-L, followed by treatment with GOH3, an antibody that blocks α6-integrin binding to the its ligand laminin (Fig. 5*B*), or control IgG. GOH3 is also routinely used as direct binding ligand to activate integrin clustering (36). Interestingly, treatment with GOH3 modestly increased the migration of BT549 cells with control pLKO. As predicted by the data thus far, silencing ADAM9 with shRNA increased cell migration compared to control (compare lane 5 to lane 1). However, treatment with GOH3 significantly increased migration (compare lane 6 to lane 5), an effect that was quantitatively rescued by expression of the shRNA-resistant mADAM9-L allele (compare lane 8 to lane 6). This demonstrates that ADAM9-L suppresses cell migration by altering integrin-mediated signals, either at the level of integrin binding, or by interacting with a parallel signaling pathway that engages in cross-talk downstream of integrin receptors.

Discussion

The ADAM family of metalloproteases controls multiple phenotypes associated with tumor progression, although the specific role of individual isoforms in tumorigenesis is poorly understood. Several ADAM isoforms, including ADAM9, have been shown to promote cell migration. In this study we identify non-redundant roles for the secreted and membrane-bound ADAM9 variants in breast cancer cell migration. Consistent with previous observations, we show that ADAM9-S can promote breast cancer cell migration in a cell-autonomous manner. Surprisingly, this phenotype is not recapitulated by ADAM9-L, which functions as a migration suppressor.

The *ADAM9 gene* (8p11.23) is located in the 8p11-12 cluster that is frequently amplified or deleted in breast cancer, with instances of abnormal copy number that correlate with reduced patient survival (37). Although ADAM9 null mice have no overt phenotypes (38), in mouse models of breast, colon and prostate carcinoma such as MMTV-PyMT, APC/Min/+, and W^{10}, overexpression of ADAM9 in neoplastic regions is detected (35). ADAM9 -/- W^{10} mice have well-differentiated tumors compared to tumors in ADAM9 +/+ W^{10} [35]. Immunohistochemical analysis of ADAM9 expression in breast tumors and cell lines support our finding that secreted ADAM9-S is expressed in breast cancer tissues (29, 30). In our studies the ADAM9-specific antibodies were unable to provide a clear detection of protein expression by immunohistochemistry or immunocytochemistry, but regardless this is the first clear identification of ADAM9-S expression in tumors and cell lines.

Breast cancer cell lines are separated into different clusters based on gene expression profiles (39). Luminal cell lines express genes associated with a more differentiated, non-invasive phenotype, while basal cell lines are less differentiated and more invasive. In a survey of gene expression profiles of 51 breast cancer cell lines, the basal gene cluster was separated into two groups, basal A and B, of which B has a stem-cell like expression profile closest to the clinical “triple-negative” tumor profile (31). Cell lines with *ADAM9* amplification and with or without ADAM9 mRNA overexpression were identified in this analysis. In our present study, we find that ADAM9-L is expressed in cells of each gene cluster, while ADAM9-S is expressed primarily in the more aggressive basal genotype (Fig. 1*C*), correlating with the finding that ADAM9-S promotes cell migration.

Our previous studies had identified ADAM9-S as a pro-migration factor that is secreted by activated liver myofibroblasts in the wound healing response (26). Tumors are sites of chronic wounding, and cancer cells adapt to the wound by increasing cell migration and angiogenesis. The success of tamoxifen treatment in recurring breast cancer with a high stromal content is correlated with elevated levels of ADAM9 (40). Similarly, ADAM9 is upregulated in the stroma surrounding invasive melanoma (41) and colon cancer (26), as well as prostate cancer cells (42, 43). These studies emphasize the importance of ADAM9 function at the tumor-stromal boundary, suggesting that ADAM9 may function downstream of paracrine factors involved in wound healing. We find that while ADAM9-S promotes cell migration via its metalloprotease activity (Fig. 2*A*), ADAM9-L functions as a migration suppressor in a manner that is independent of

proteolytic activity (Fig. 4*B*). ADAM9-S may proteolyze specific substrates on the cell surface or ECM that are not targeted by ADAM9-L as its localization is restricted by membrane tethering. Consistent with this, cells expressing ADAM9-S have a higher rate of migration than cells exposed to a more diffuse exogenously-added ADAM9-S (Fig. 2*A* and 2*B*), indicating that ADAM9-S might function in a cancer cell autonomous manner rather than acting on the tumor stroma. Regardless, the opposing functions of ADAM9-L and ADAM9-S in cell migration suggest that the two splice variants control tissue homeostasis, with isoform-specific functions in response to signals both from neighboring tumor and stromal cells. A recent shRNA screen for cell migration enhancers and suppressors identified ADAM9 as a migration suppressor in non-tumorigenic MCF10A epithelial cells, consistent with our findings (44). Surprisingly, we find that the ADAM9-L metalloprotease domain is dispensable for this phenotype, whereas the cytoplasmic and disintegrin domains are required, indicative of a role for membrane localization and cytoplasmic signaling.

The disintegrin domain of ADAM9 influences cell migration in a cell type-dependent manner. Keratinocytes adhering to recombinant ADAM9-L disintegrin domain exhibit increased migration (25), a result also observed with fibroblasts adhering to recombinant ectodomain (45). In contrast, recombinant disintegrin domain inhibits the adhesion of platelets to collagen I (46), and interacts with multiple integrins resulting in ERK (extracellular regulated kinase) phosphorylation, activation of p38MAPK, $cPLA_2$ and MMP-9 synthesis (25, 27). In breast carcinoma cells, antibody binding to α6-integrin enhances migration, a phenotype that is significantly enhanced in the absence of ADAM9 expression (Fig. 5*B*). ADAM9-L may control the localization of integrins or Syndecans to specific lipid rafts (47), or alternatively modulate endocytosis of cell surface receptors. ADAM9 has also been shown to interact with and promote the recycling of E-cadherin in colon cancer cells, and this may represent a metalloprotease independent mechanism for ADAM9-L (48).

The opposing roles for ADAM9-S and ADAM9-L as migration enhancers and suppressors in breast cancer cells raises the question as to how the expression profile of each splice variant is regulated. This is predicted to have a major impact in tumor progression as cells overexpressing ADAM9-L would presumably have a reduced migration phenotype. Conversely, any tumors in which ADAM9-S is the predominant isoform would be predicted to have aggressive invasive and metastatic characteristics. Although presently unknown, it is highly likely that one important mechanism is the control of alternative splicing of the ADAM9 message.

In summary, our results show that the secreted and membrane-tethered isoforms of ADAM-9 have opposing functions in breast cancer cell migration. The expression of both isoforms in breast cancer tumors and stromal suggests a model in which the secreted ADAM9-S acts as a migratory stimulant, while ADAM9-L tethered to the plasma membrane binds cell surface proteins and mediates localization and signaling. One important implication from these findings is that isoform-specific functions of secreted and membrane-tethered proteases exist which has obvious consequences for the development of small molecule inhibitors for therapeutic intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

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Figure Legends

Figure 1 Expression of ADAM9 Isoforms in Breast Cancer Cell Lines and Tissues

*A***,** A schematic representation of the membrane-bound (ADAM9-L) and secreted (ADAM9-S) isoforms of ADAM9.

*B***,** HEK293T cells transiently transfected with a vector control (pCDNA4), ADAM9-L.myc and ADAM9-S.myc were lysed and proteins resolved on an 8% SDS-PAGE gel. Equivalent lanes were blotted with anti-myc to validate protein expression. Equivalent lanes were blotted with ADAM9-L and ADAM9-S specific antibodies.

C, a panel of cell lines representing breast cancer subtypes were used for immunoblotting with ADAM9-L C-terminal specific antibody, ADAM9-S C-terminal specific antibody, and β-actin. # denotes overexpression of ADAM9 and amplified 8p11-12 , * denotes amplified 8p11.12.

*D***,** SDS-extracted lysates from frozen tumor blocks were immunoblotted with anti-ADAM9-S antibody. BT549 cell lysate was used as control. All results are representative of at least three independent experiments.

Figure 2 ADAM9-S Promotes Breast Cancer Cell Migration

A, migration of BT549 cells overexpressing ADAM9-L and -S. Cells were migrated for 4 h in Transwells. Expression of transfected proteins was confirmed with anti-ADAM9-L C-terminal specific antibody or anti-myc. ADAM9-L is denoted by arrows. Overexpressed murine ADAM9-L migrates slower than endogenous human ADAM9-L. *, $p< 0.001$, calculated using Anova.

B, migration of BT549 cells stimulated with exogenous ADAM9-S. Cells were migrated for 16 h in Transwells in which the bottom chamber contained media collected from HEK293T cells expressing pCDNA control, ADAM9-S or ADAM9S.EA. Expression of ADAM9-S was confirmed by anti-myc immunoblotting. *, $p< 0.05$, calculated using Anova. All results are mean +/- SD of triplicate measurements of at least three independent experiments.

Figure 3 ADAM9 Silencing Increases Cell Migration

A, BT549 cells were transfected with β-galactosidase alone, or in conjunction with non-targeting siRNA oligonucleotide, or ADAM9 siRNA oligonucleotide pool. Cells were migrated for 16 h. *, $p<0.01$, calculated using Anova. Silencing was confirmed by immunoblotting using ADAM9-L C-terminal specific antibody and β-actin.

B, BT549 cells infected with ADAM9 shRNA.1 and shRNA.2 were lysed and immunoblotted against ADAM9-L and ADAM9-S. ADAM9-S was immunoprecipitated from equal cell lysates prior to immunoblotting.

C, BT549 cells were infected with control or ADAM9 shRNA.1 lentiviruses before transfection of pCDNA control, ADAM9-L, ADAM9-S and ADAM9S.EA. Cells were migrated for 4 h. *, $p < 0.05$, ** $p<0.001$, calculated using Anova. ADAM9-L immunoblot confirms silencing. ADAM9-S expression is detected with anti-Myc.

D, BT549, CAMA-1 and ZR75-1 cells were infected with control or ADAM9 shRNA.1 or shRNA.2 lentiviruses. Selected cells were migrated in uncoated Transwells for 4 h (BT549) or 16 h. *, $p<0.03$, calculated using Anova. Silencing is confirmed by immunoblot with ADAM9-L C-terminal antibody. In all experiments cell counts were normalized and results shown are the mean +/- SD of triplicate measurements of at least three independent experiments.

Figure 4 ADAM9-L Suppresses Cell Migration in a Metalloprotease-independent Manner

A, schematic representation of wild type ADAM9 isoforms and functional domain mutations used to assess the role of each domain in cell migration.

B, BT549 cells were infected with control or A9shRNA.1 lentiviruses before transfection with pCDNA control, mADAM9-L wild type or mADAM9L.EA post-selection. Cells were migrated in uncoated Transwells for 4 h. *, $p<0.01$, calculated using Anova. Cell lysates were immunoblotted against actin and ADAM9-L to detect both murine (upper arrows) and human (lower arrows) variants.

C, BT549 cells were infected with control or A9shRNA.1 lentiviruses before transfection with pCDNA control, mADAM9-L wild type or mADAM9L.Δcyt post-selection. Cells were migrated in uncoated Transwells for 4 h. . *, $p<0.05$, *** $p<0.001$, calculated using Anova. ADAM9-L expression is detected with anti-Myc, ADAM9 silencing is detected with anti-ADAM9-L, and actin is control. ns, non-specific immunoreactive protein. In all experiments cell counts were normalized to transfection efficiency and results are the mean +/- SD of triplicate measurements of at least three independent experiments.

Figure 5 The ADAM9-L Integrin Binding Activity Modulates Cell Migration

A, HEK293T cells were transfected with wildtype mADAM9-L, point mutant mADAM9L.TCE.myc, and mADAM9.Δdis.myc. Lysates were immunoblotted with ADAM9-L C-terminal antibody.

B, BT549 cells infected with control PLKO or ADAM9shRNA.1 virus were transfected with pCDNA control or mADAM9L wildtype constructs. Cells were resuspended in 0.01% BSA in PBS with 5µg/mL GOH3 antibody and incubated for 10 min prior to a 4 hr migration assay. Lysates were immunoblotted with ADAM9-L C-terminal antibody to confirm silencing and expression. All results shown are mean +/- SD of triplicate measurements of two independent experiments. *p* values were calculated using Anova *, $p<0.05$, ***, $p<0.001$.

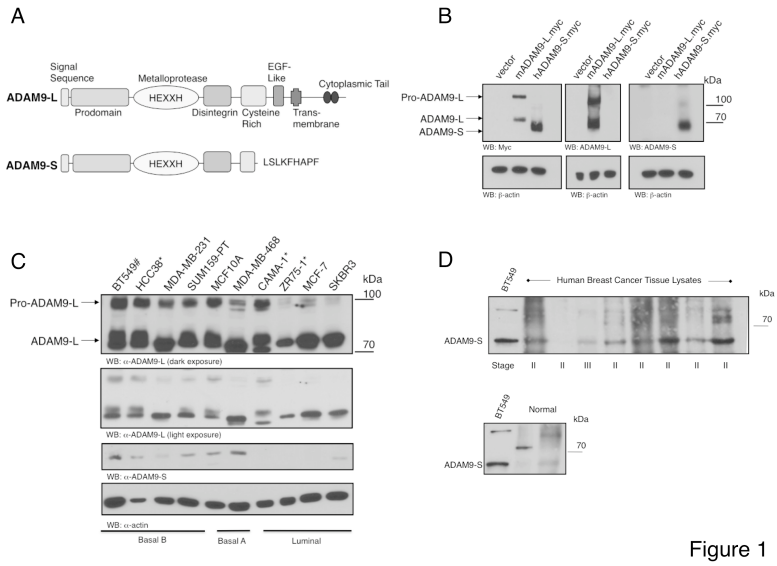
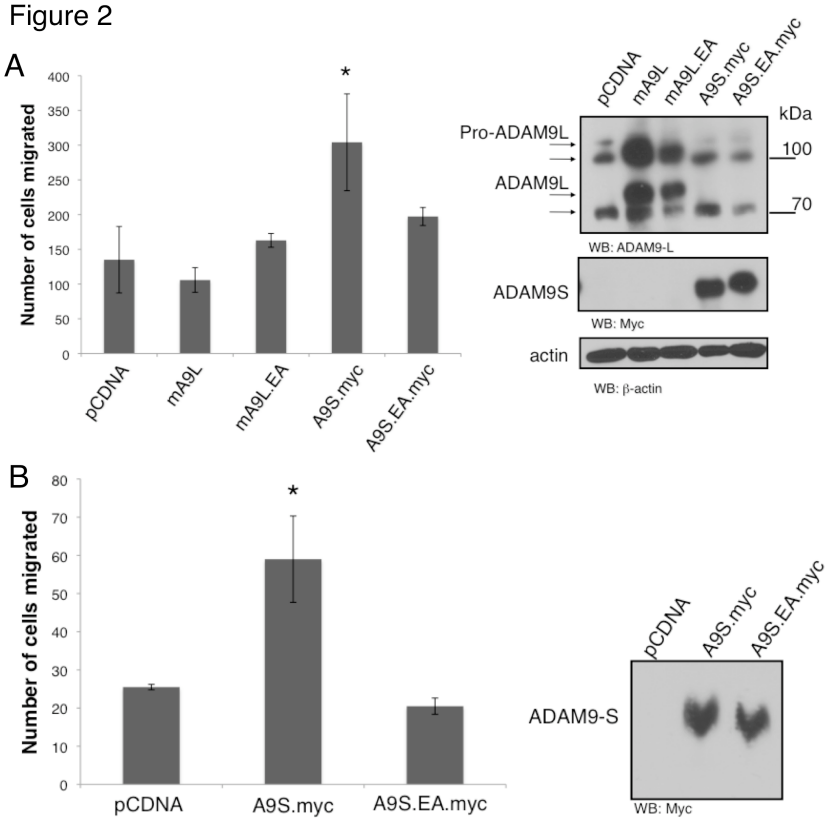
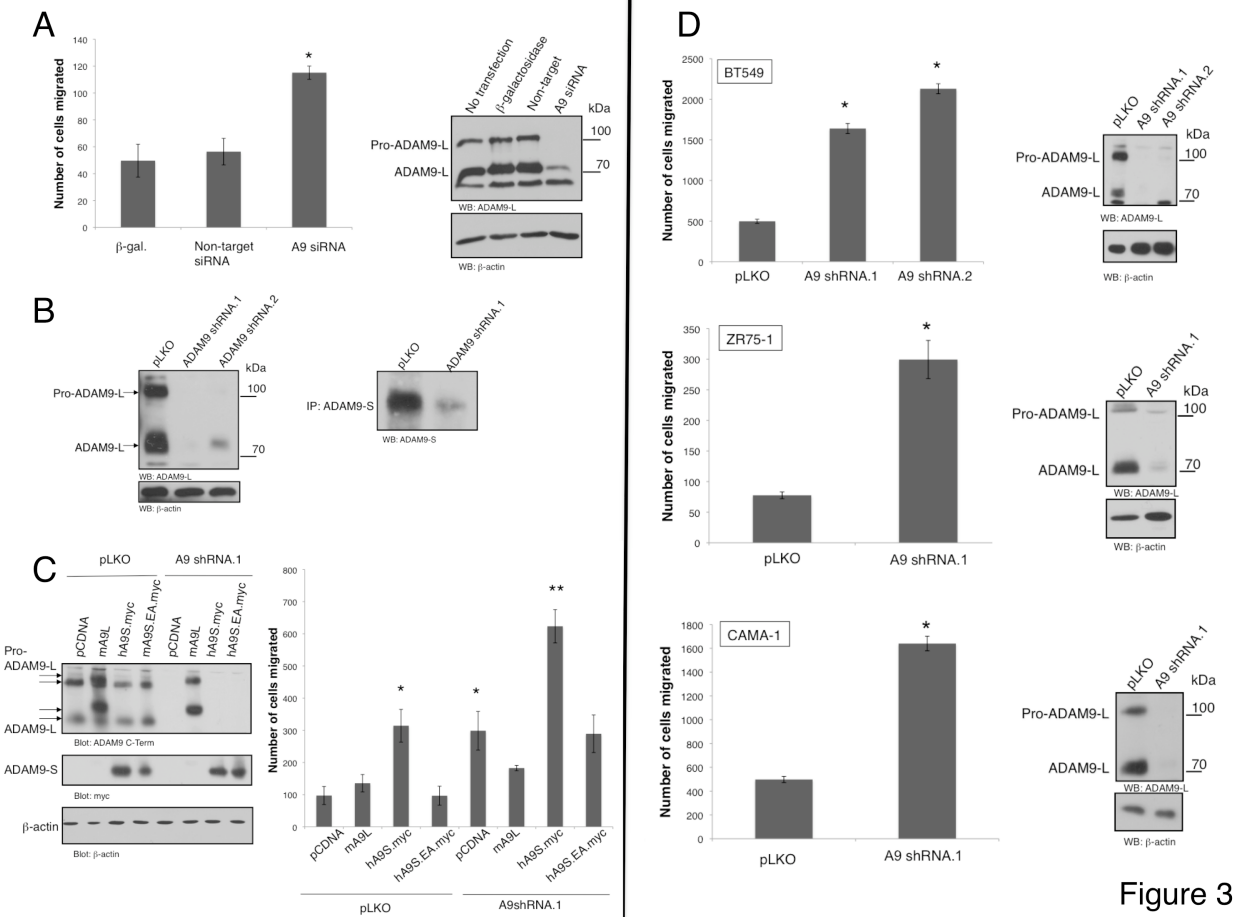


Figure 1

Figure 2




A
Number of cells migrated
β-gal.
Non-target siRNA
A9 siRNA
No transfection
β-galactosidase
Non-target
A9 siRNA
kDa
Pro-ADAM9-L
ADAM9-L
100
70
WB: ADAM9-L
WB: β-actin
B
pLKO
ADAM9 shRNA.1
ADAM9 shRNA.2
IP: ADAM9-S
WB: ADAM9-S
C
pLKO
A9 shRNA.1
pCDNA
mA9L
hA9S.myc
mA9S.EA.myc
hA9S.myc
hA9S.EA.myc
Pro-ADAM9-L
ADAM9-L
Blot: ADAM9 C-Term
ADAM9-S
Blot: myc
β-actin
Blot: β-actin
Number of cells migrated
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D
BT549
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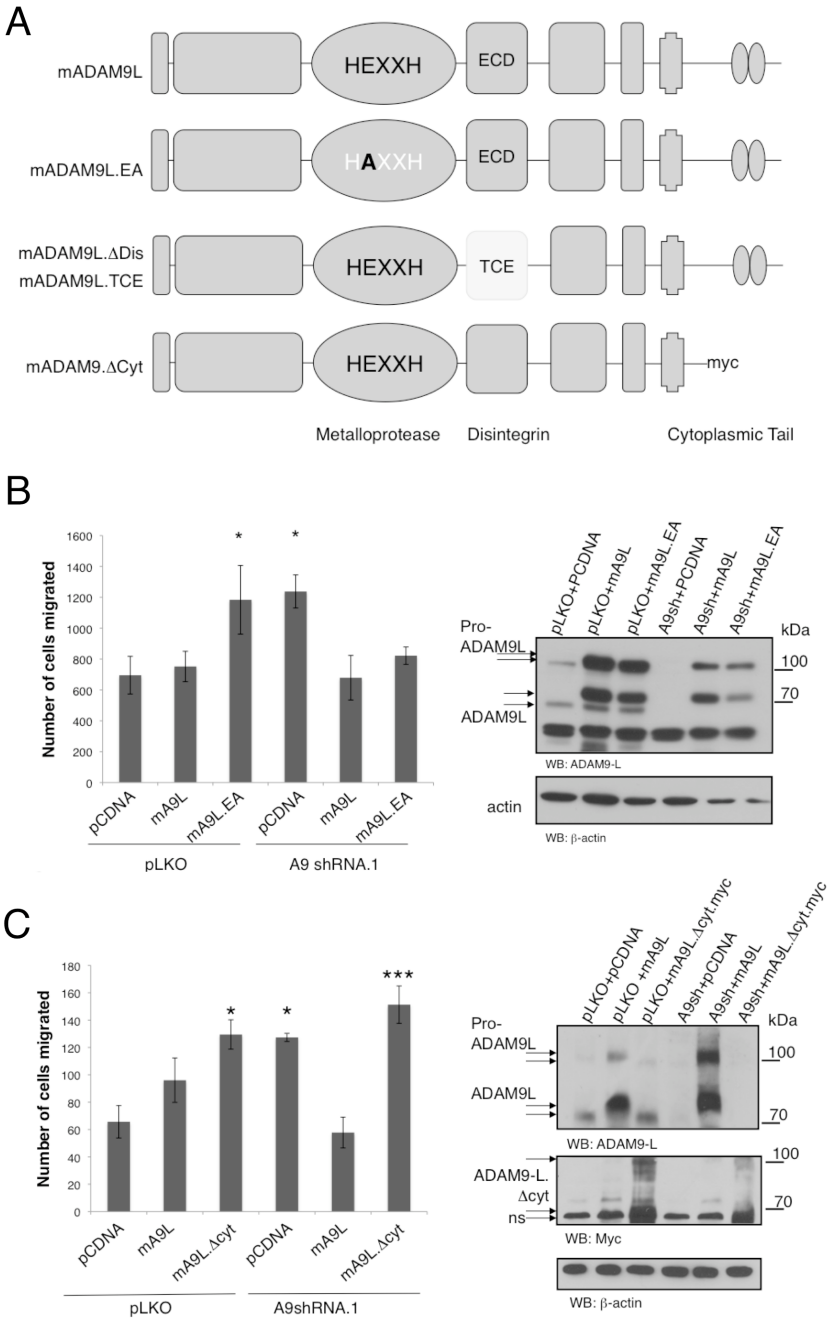
CAMA-1
pLKO
A9 shRNA.1
A9 shRNA.2

Figure 3


A
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HEXXH
ECD
mADAM9L.EA
HAXXH
ECD
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mADAM9L.TCE
HEXXH
TCE
mADAM9.ΔCyt
HEXXH
myc
Metalloprotease
Disintegrin
Cytoplasmic Tail
B
Number of cells migrated
pCDNA
mA9L
mA9L.EA
pCDNA
mA9L
mA9L.EA
pLKO
A9 shRNA.1
pLKO+PCDNA
pLKO+mA9L
pLKO+mA9L.EA
A9sh+PCDNA
A9sh+mA9L
A9sh+mA9L.EA
Pro-ADAM9L
ADAM9L
kDa
100
70
WB: ADAM9-L
actin
WB: β-actin
C
Number of cells migrated
pCDNA
mA9L
mA9L.Δcyt
pCDNA
mA9L
mA9L.Δcyt
pLKO
A9shRNA.1
pLKO+pCDNA
pLKO +mA9L
pLKO+mA9L.Δcyt.myc
A9sh+pCDNA
A9sh+mA9L
A9sh+mA9L.Δcyt.myc
Pro-ADAM9L
ADAM9L
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70
WB: ADAM9-L
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ns
100
70
WB: Myc
WB: β-actin

Figure 4

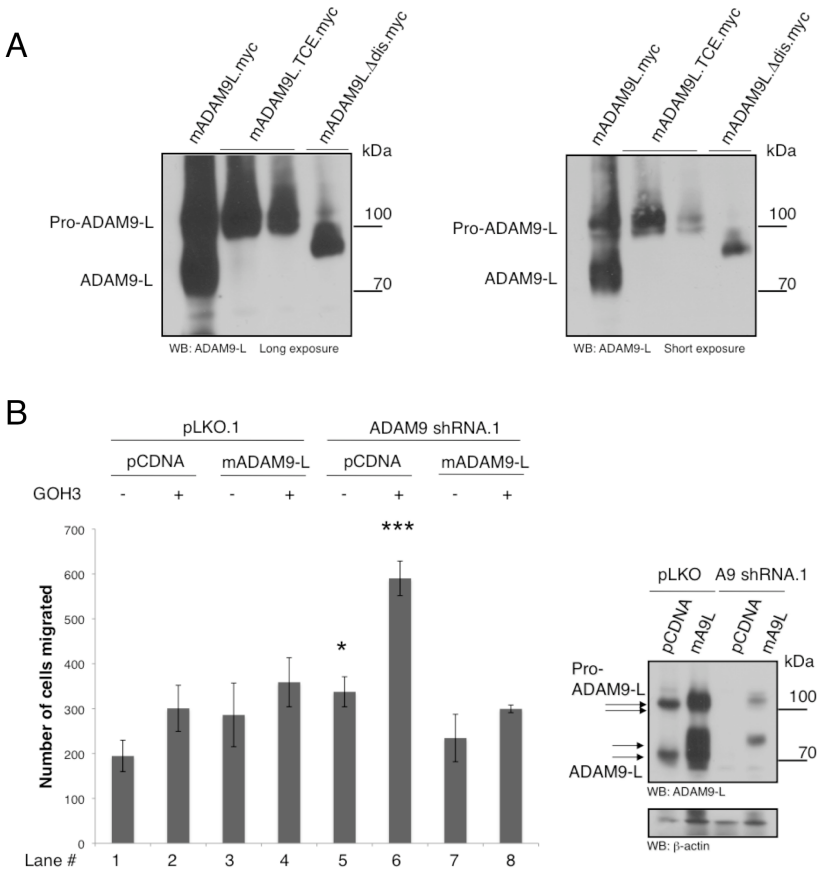
A
mADAM9L.myc
mADAM9L.TCE.myc
mADAM9L.Δdis.myc
kDa
Pro-ADAM9-L
ADAM9-L
100
70
WB: ADAM9-L Long exposure
WB: ADAM9-L Short exposure
B
pLKO.1
ADAM9 shRNA.1
pCDNA
mADAM9-L
GOH3
Number of cells migrated
Lane #
pLKO
A9 shRNA.1
pCDNA
mA9L
Pro-ADAM9-L
ADAM9-L
WB: ADAM9-L
WB: β-actin

Figure 5